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Quantitation of tigecycline, a novel glycyclcycline, by liquid chromatography

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Abstract

An ion-paired HPLC assay was developed to determine tigecycline (GAR-936) concentrations in Hank's balanced salts solution, tigecycline intra-cellular concentrations in human polymorphonuclear neutrophils (PMNs) and tigecycline concentrations in human serum. Minocycline was used as internal standard, 5% trichloroacetic acid was added to lyse PMNs and also precipitate proteins in PMNs and serum. The top aqueous layer was aspirated for HPLC assay. The chromatograms were performed with a reversed-phase C18 column with UV detector. The mobile phase consisted of acetonitrile, phosphate buffer (pH 3) and 1-octanesulfonic acid at a flow rate of 1 ml/min. Good linearity and recovery were achieved over the range of standard curves. The relative standard deviations of three quality controls for intra- and inter-day precision were less than 6.4%, and the relative errors of the intra- and inter-day accuracy were less than 7.0%. Tigecycline in Hank's buffer, PMNs and serum was stable under different test conditions. This new liquid chromatography assay is a simple, accurate and reproducible method for determining tigecycline in different matrix.

Keywords: Tigecycline; HPLC

1. Introduction

Tigecycline (GAR-936, Fig. 1A), a novel glycyclcycline, is a semisynthetic intravenous antibiotic. Tigecycline binds to bacterial 30S ribosomal subunit to inhibit protein synthesis in bacteria, and has shown a broad-spectrum of antibacterial activity against anaerobic bacteria, Gram-negative, and Gram-positive including methicillin-resistant *Staphylococcus aureus* [1]. Tigecycline also has verified effective in vivo efficacy in different animal models [2–5]. Human phase I and II clinical trials have been completed for tigecycline, phase II studies have been conducted in patients with complicated skin and skin structure infections and complicated intra-abdominal infections [6].

To date no HPLC method has been reported to analyze tigecycline. A new HPLC assay was developed to determine tigecycline concentrations in Hank's balanced salts solution (HBSS), tigecycline intra-cellular concentrations in human polymorphonuclear neutrophils (PMNs) and tigecycline concentrations in human serum.

2. Experimental

2.1. Chemicals and reagents

Tigecycline was provided by Wyeth (Pearl River, NY). Minocycline (hydrochloride salt), 1-octanesulfonic acid, trichloroacetic acid, sodium dihydrophosphate monohydrate and Hank's balanced salts solution (pH 7.2–7.6) were purchased from Sigma (St. Louis, MO). Phosphoric acid was obtained from J.T. Baker (Phillipsburg, NJ). HPLC grade acetonitrile (Mallinckrodt Baker, Paris, KY) was used without further purification. Deionized water was obtained from a Milli-Q Plus analytical deionization system (Bedford, MA).

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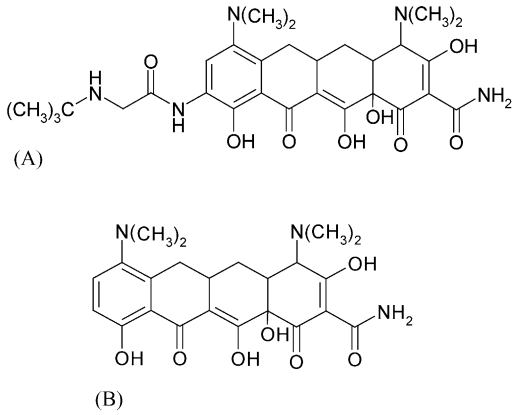

Fig. 1. The chemical structure of (A) tigecycline and (B) minocycline, the internal standard.

2.2. Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a 626 gradient pump (Waters, Milford, MA, USA), a 600S controller (Waters, Milford, MA), a 717 plus autosampler (Waters, Milford, MA), and LDC SM4000 programmable wavelength UV detector (LDC/Milton Roy, Riviera Beach, FL). The separation was carried on a Phenomenex Luna C18 150 mm × 4.5 mm column (5 μm) (Torrance, CA) preceded with a μBondapak C18 Guard-Pak precolumn (Waters, Milford, MA). EZChrom Elite chromatography data system (Scientific software, San Ramon, CA) was employed to quantify the peak areas.

The mobile phase was composed of 25:75 (v/v) acetonitrile–phosphate buffer (pH 3.0, 0.023 M) with 4 mM 1-octanesulfonic acid. The mobile phase flow rate was 1 mL/min. The UV detector was set at 244 nm with a sensitivity of 0.002 aufs for HBSS samples, and 350 nm with a sensitivity of 0.001 aufs for human PMN and serum samples. The running time for one sample was 10 min. All chromatographic procedures were performed at ambient temperature.

2.3. Preparation of standards and controls

Tigecycline stock solution (2000 μg/mL) was made in volumetric flasks with deionized water. The internal standard solution, minocycline (1000 μg/mL), was prepared in deionized water, then diluted in phosphate buffer (pH 4, 0.1 M) to make 30 μg/mL.

Human PMNs were separated from blood, and reconstituted at HBSS to attain cell concentration at 5×10^6 cells/mL. The PMN suspension was frozen at −20 °C before use. Drug-free human blood was collected and centrifuged at 2400 × *g* for 10 min. The pooled blank serum was stored at −20 °C prior to use. Tigecycline was spiked in HBSS, human serum to make six standard solutions (0.05, 0.3, 0.6, 2.0, 3.0 and 5.0 μg/mL) and three quality controls (0.1, 1.0 and 4.0 μg/mL). For human PMN suspension tigecycline was spiked to make 6 standard solutions (0.05, 0.2, 0.4, 0.8, 1.0 and 3.0 μg/mL) and three quality controls (0.1, 0.6 and 2.0 μg/mL).

2.4. Sample extraction

Aliquots (200 μL) of standards, controls, or unknown samples, and 30 μL of internal standard solution were transferred into a test tube. Ten microliters of 5% trichloroacetic acid was added to HBSS samples. For PMN samples, 100 μL of 5% trichloroacetic acid was added to lyse cells and precipitate proteins. Serum samples was deproteinized by the addition of 300 μL of 5% trichloroacetic acid. After vigorous vertex and centrifuge, the upper aqueous layer was aspirated for the HPLC assay.

2.5. Quantification

Quantitative analyses were performed using the internal standard method. Standard curves were generated by plotting the ratio of the peak area of tigecycline to that of internal standard against standard solution concentrations. Weighted (1/concentration) least square linear regression analyses were applied to obtain the linear regression equation. Linearity of the standard curves was assessed with the intercept, slope and correlation coefficient (*r*). Concentrations of quality controls and unknown samples were calculated by applying the linear regression equation of the standard curve.

2.6. Method validation

Five samples with 0.05 μg/mL tigecycline in HBSS, PMNs and serum were used to evaluate the lower limits of quantification (LLQ) of the assay. The precision of LLQ samples was determined by the relative standard deviations (R.S.D.) of mean values; the accuracy of LLQ samples was assessed by the relative error from the theoretical concentrations. Three quality controls with low, middle and high concentrations were used to evaluate the assay recovery. Values of percentage recoveries of tigecycline were estimated by comparing the peak area ratio of tigecycline to the internal standard in human PMNs and serum to that in aqueous solution following the above-described extraction procedure. The recovery of internal standard minocycline was estimated by comparing the peak area of minocycline in human PMNs and serum to that in aqueous solution.

The precision and accuracy of this method were evaluated by the three quality controls. The assay precision was assessed by the relative standard deviation of mean value from the theoretical concentrations; the assay accuracy was assessed by the relative error of the back-calculated concentration from the theoretical concentrations. The stability of tigecycline was inspected during the storage steps and extraction steps. Since tigecycline in HBSS was unstable at room temperature for 4 h, the stability was tested while 10 μL 5% trichloroacetic acid was added to 200 μL tigecycline HBSS and 100 μL 5% trichloroacetic acid was added to 200 μL

tigecycline PMN suspension in HBSS. The counter top storage stability was determined after samples sitting at room temperature for 4 h, the autosampler stability was evaluated the extracted samples sitting at the 10 °C autosampler for 8 h, and the freeze–thaw stability was determined over two freeze–thaw cycles. In addition, the stability of tigecycline in HBSS was evaluated at 37 °C in a water bath for tigecycline uptake study in human PMNs.

3. Results and discussion

3.1. Chromatography

Several mobile phases based on acetonitrile, methanol and phosphate buffer with different pH were tried. It was noticed that the pH of the mobile phase affected the retention time of tigecycline. The higher the pH of the phosphate buffer, the longer the retention time of tigecycline. Peak tailing was observed when the phosphate buffer was at pH 7.1, no peak tailing was observed when the phosphate buffer pH was changed to 5.5. In previous reported HPLC assays for doxycycline (a tetracycline derivative), the sensitivity of the assay depended on the pH of mobile phase and extracted samples [7,8]. We observed the same phenomena with tigecycline, and the high sensitivity was attained when phosphate buffer at pH 3 was used in the mobile phase. Since tigecycline retention time was too short (1.5 min) as the mobile phase consisted of acetonitrile and phosphate buffer at pH 3 (v/v, 20:80) at the flow rate of 1 mL/min, an ion pair agent 1-octanesulfonic acid was added to the mobile phase to prolong the retention time of tigecycline.

Under the previous described chromatographic conditions, no endogenous peaks interfered with tigecycline and minocycline in HBSS, human PMNs and serum. A good resolution was obtained between tigecycline and minocycline. Fig. 2A and B represents the typical chromatograms of blank HBSS and tigecycline with internal standard in HBSS. Fig. 3A and B represents the typical chromatograms of blank human PMNs and tigecycline with internal standard in human PMNs. Fig. 4A and B represents the typical chromatograms of blank human serum and tigecycline with internal standard in human serum. The retention times for tigecycline and minocycline were 5.1 and 7.3 min, respectively. Total running time for one sample was 10 min.

3.2. Linearity

The linearity of the assay was assessed with standard curves ranging 0.05–4 μg/mL in HBSS, 0.05–3 μg/mL in human PMNs, and 0.05–4 μg/mL in human serum. All three standard curves with six standard solutions were generated by plotting the peak area ratio of tigecycline to internal standard versus the theoretical concentrations. Table 1 presents the summary data of correlation coefficient, slope and intercept of the standard curves of tigecycline in HBSS, human

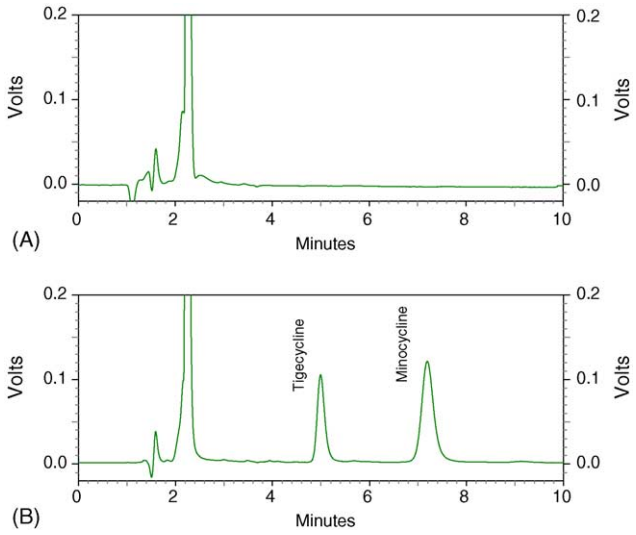

Fig. 2. Chromatograms for (A) blank HBSS and (B) 2 μg/mL tigecycline spiked in HBSS.

PMNs and serum. The correlation coefficient (r) of the standard curves were larger than 0.996. Values of the correlation coefficient for all the standard curves are satisfactory.

3.3. Lower limit of quantification

The LLQ of tigecycline in HBSS, human PMNs and serum were chosen as the concentrations used for the lowest concentration in the standard curves. The LLQ was 0.05 μg/mL for tigecycline in HBSS, human PMNs and serum. The precision and accuracy of LLQ of tigecycline were 6.13% and 12.44% in HBSS, 4.98% and 4.73% in PMNs, and 5.17% and 1.85% in serum. It is adequate to take into account the concentration of tigecycline 0.05 μg/mL in analyzing unknown samples.

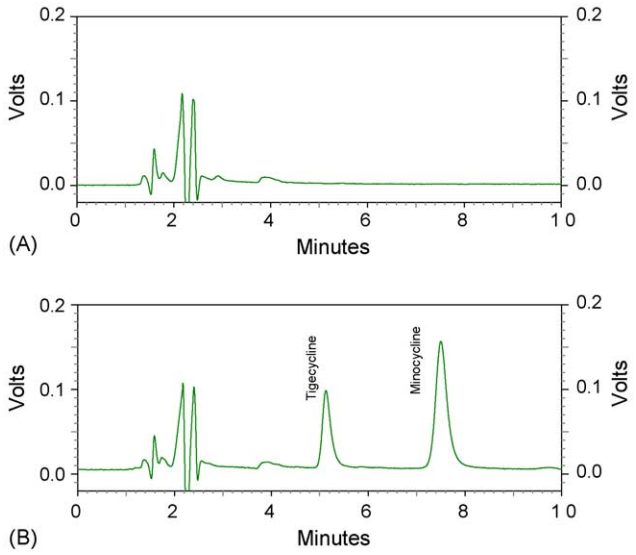

Fig. 3. Chromatograms for (A) blank human PMNs and (B) 2 μg/mL tigecycline spiked in human PMNs.

Table 1
Assay linearity for tigecycline in HBSS, human PMNs and serum

Matrix	Correlation coefficient (mean ± S.D.)	Slope (mean ± S.D.)	Intercept (mean ± S.D.)
HBSS solution[a]	0.9991 ± 0.0010	0.4089 ± 0.0537	−0.0090 ± 0.0041
Human PMNs[b]	0.9995 ± 0.0004	0.2221 ± 0.0102	0.0046 ± 0.0067
Human serum[c]	0.9989 ± 0.0012	0.1962 ± 0.0091	−0.0005 ± 0.0035

[a] $n = 15$.
[b] $n = 17$.
[c] $n = 6$.

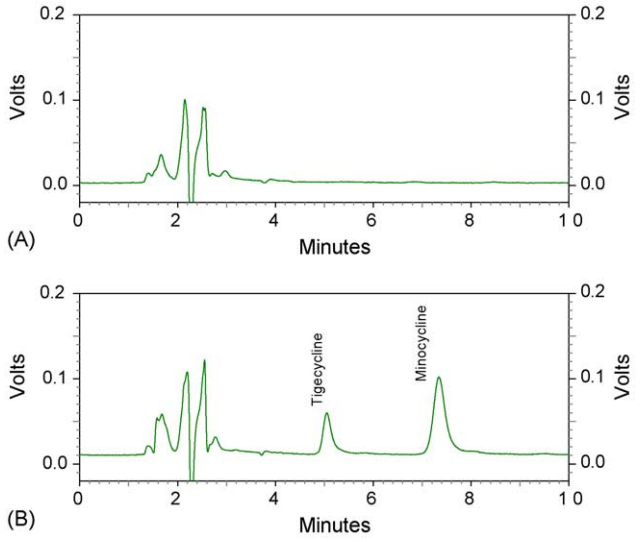

Fig. 4. Chromatograms for (A) blank human serum and (B) 2 μg/mL tigecycline spiked in serum.

3.4. Precision and accuracy

The inter- and intra-day precision and accuracy were estimated by analyzing quality controls in HBSS, human PMNs and serum with three levels of concentrations (low, middle, and high). The summary data of inter- and intra-day precision and accuracy of tigecycline in three matrixes is shown in Tables 2–4. The inter-day precision of three quality controls of tigecycline in HBSS, human PMNs and serum was from 3.03% to 6.25%, the intra-day precision of tigecycline was from 0.74% to 6.39%. The inter-day accuracy of three quality controls of tigecycline in HBSS, human PMNs and serum was from 0.09% to 3.90%, the intra-day accuracy of tigecycline was from 0.58% to 6.95%. In all of quality controls, R.S.D. and relative errors of tigecycline in three matrixes were less than 7%. The precision and accuracy of this assay were all within the acceptable range for bioanalytical quantification.

Table 2
Precision and accuracy of the assay for tigecycline in HBSS

	Theoretical concentration (μg/ml)		
	Low (0.1)	Medium (1.0)	High (4.0)
Intra-day			
Mean[a]	0.093	0.971	4.055
S.D.	0.002	0.062	0.077
R.S.D. (%)	2.09	6.39	1.90
Relative error (%)	6.95	2.95	1.36
Inter-day			
Mean[b]	0.098	1.039	4.063
S.D.	0.004	0.035	0.219
R.S.D. (%)	4.04	3.35	5.39
Relative error (%)	2.41	3.90	1.57

[a] $n = 10$.
[b] $n = 15$.

Table 3
Precision and accuracy of the assay for human PMNs

	Theoretical concentration (μg/ml)		
	Low (0.1)	Medium (0.6)	High (2.0)
Intra-day			
Mean[a]	0.101	0.597	2.085
S.D.	0.005	0.010	0.015
R.S.D. (%)	4.95	1.76	0.74
Relative error (%)	0.90	0.58	4.23
Inter-day			
Mean[b]	0.097	0.601	2.068
S.D.	0.005	0.028	0.066
R.S.D. (%)	4.96	4.73	3.21
Relative error (%)	3.03	0.24	3.38

[a] $n = 10$.
[b] $n = 17$.

3.5. Recovery

The mean percentage recoveries of tigecycline in human PMNs and serum were calculated as 112.65 ± 7.31 and 111.96 ± 4.40, respectively ($n = 9$). The mean recovery

Table 4
Precision and accuracy of the assay for tigecycline in human serum

	Theoretical concentration (μg/ml)		
	Low (0.1)	Medium (1.0)	High (4.0)
Intra-day			
Mean[a]	0.099	1.049	4.266
S.D.	0.004	0.049	0.100
R.S.D. (%)	4.28	4.67	2.35
Relative error (%)	0.70	4.90	6.66
Inter-day			
Mean[b]	0.0997	0.999	4.124
S.D.	0.006	0.030	0.258
R.S.D. (%)	5.72	3.03	6.25
Relative error (%)	0.32	0.09	3.10

[a] $n = 10$.
[b] $n = 6$.

of the internal standard in human PMNs and serum were 105.04 ± 3.76 and 87.18 ± 3.65, respectively ($n = 9$). The extraction efficiency of tigecycline and minocycline from human PMNs and serum was sufficient, larger than 87%.

3.6. Stability

The stability of tigecycline in HBSS, human PMNs and serum was inspected by analyzing the three quality controls under different conditions. After counter top storage at room temperature, tigecycline was stable in HBSS, human PMNs and serum for 4 h, the percent recovery ranged from 93.0% to 107.2%. No significant degradation was observed in the extracted samples in the three matrixes after sitting in autosampler at 10 °C for 8 h, losses less than 9.3% for tigecycline was observed. The results indicated that tigecycline is stable during the sample processing and analyzing procedure.

After two freeze–thaw cycles, less than 8.5% of tigecycline was decomposed. That means, two freeze–thaw cycles can be tolerated with losses less than 10% for tigecycline in HBSS, human PMNs and serum. Moreover, to ensure HBSS as the appropriate media for tigecycline uptake study in human PMNs, the stability of tigecycline in HBSS was tested at 37 °C for 4 h. Interestingly, tigecycline at 4 μg/mL was stable in HBSS at 37 °C for 4 h, no degradation was observed. Also we observed that after pH was adjusted to 2, tigecycline in HBSS was stable at 37 °C for 4 h. Overall, tigecycline is stable under our test conditions.

In conclusion, this paper describes a new liquid chromatographic analysis of tigecycline in HBSS, human PMNs and serum. The inter- and intra-day precision and accuracy of the assay is less than 7%, which ensure precise measurements of tigecycline in different matrix. This assay is simple, specific and reproducible, and has been successfully applied to monitor tigecycline penetration in and out human PMNs.

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